BACTERIAL CHARACTERISATION AND PATHOGENICITY

Expression of *Bacteroides fragilis* virulence markers *in vitro*

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Bacteroides fragilis isolates from intestinal and non-intestinal infections, normal flora and the environment were examined for properties linked with interactions among cells *in vitro*. Different adhesion molecules were detected in agglutination assays with human erythrocytes and tests for auto-agglutination and adherence to human colon carcinoma cells (HT29). There was no correlation between these properties, indicating that independent molecules are involved. Treatment with trypsin, heat or EDTA inhibited agglutination and adherence, suggesting that these molecules are proteins. The lack of correlation with the origin of the strains did not permit any of these activities to be recognised as virulence markers. The expression of fragilysin, a protease associated with damage to intestinal cells and bacterial translocation, was examined. Only those strains from patients with diarrhoea expressed this protease activity in assays with HT29 cells and this was confirmed by specific PCR for the *bft* gene. The activity of fragilysin as an enterotoxin was confirmed in the rabbit intestinal ligated loop assay. The association of this property only with strains from intestinal infections indicates that it is too early to suggest this protease as a determinant factor of *B. fragilis* invasiveness.

Introduction

Bacteroides fragilis is considered to be the most virulent species in the genus *Bacteroides* because of its virulence factors and its predominance among isolates from infections, despite its presence as only a small component of the normal intestinal microflora [1]. Adhesion properties, demonstrated in assays of haemagglutination and adherence to cell cultures, have been related to virulence. Surface structures such as the polysaccharide capsule [2, 3], fimbriae [4], microcapsule and outer membrane non-fimbrial proteins [5] have been linked with adhesion to host cells, but without precise definition of the mechanisms. *B. fragilis* also produces an enterotoxin that causes diarrhoea in animals [6] and has been associated with diarrhoea in man [7]. The toxin has been purified and characterised as a zinc-metalloprotease [8], with activity against tight junction proteins, disrupting the intestinal epithelial barrier [9]. Its activity has been linked with diarrhoea and fluid loss, and also to bacterial translocation from the intestinal environment. Some authors have des-

cribed toxin expression in strains isolated from non-intestinal infections [10]. This protease has now been named fragilysin and is encoded by the *bft* gene. In the present study, *B. fragilis* isolates from different origins were compared with clinical isolates in relation to their adhesive properties, the presence of the *bft* gene and fragilysin production, with the aim of detecting *in vitro* possible virulence markers for the species.

Materials and methods

Bacterial isolates and culture conditions

Fifty-eight isolates of *B. fragilis* from different origins were analysed. They were isolated from human non-intestinal infections (20), human intestinal microflora (14), animal intestinal microflora (5) and polluted aquatic environment (11). Four human intestinal isolates, previously defined as enterotoxigenic (078320 and 079298-3) and non-enterotoxigenic (283-2-1 and 077225-2) and two animal intestinal isolates (Bd1 and 865193-4), given by Dr Lyle Myers (Montana University, USA) were included. Two reference strains obtained from the American Type Culture Collection, Rockville, MD, USA (ATCC 25285 and ATCC 23745) were also studied. One *Escherichia coli* LT-producing strain (40 T), given by Dr Beatriz Guth (Escola

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Paulista de Medicina, São Paulo, Brazil) was used as positive control in rabbit intestinal ligated loop assays. Isolation and identification were performed by conventional methods, with PRAS medium [11] and the strains were stored in skim-milk 10% at -20°C .

Haemagglutination and auto-agglutination assays

Organisms grown in BHI-PRAS [11] for 18 h at 37°C were pelleted by centrifugation, washed and resuspended in 0.01 M phosphate-buffered saline (PBS) pH 7.2, to a concentration of $c. 3.0 \times 10^{10}$ cfu/ml. Fifty μl of the bacterial suspension were serially diluted in two-fold series in a microtitration tray and 50 μl of the human A⁺ erythrocyte suspension (1%) were added to each well, except in one, for the auto-agglutination assay. The microtitration tray was shaken gently for 1 min at room temperature and then was read after incubation at 4°C for 60 min. An extra incubation, overnight at 4°C , was also done. Haemagglutination activity (HA) titres were expressed as the highest bacterial dilution that showed haemagglutination activity. The auto-agglutination activity (AA) was evaluated in a scale ranging from 1+ to 4+ [12].

Haemagglutination and auto-agglutination inhibition assays

The haemagglutination and auto-agglutination assays were done as described above, with untreated suspensions as controls and test suspension treated as follows. The bacterial suspension were heated at 100°C for 30 min and 80°C for 1 h. Assays were done with the addition of 50 μl of a 2.5 mM EDTA solution, pH 7.8, to each well. Pelleted cells were resuspended in 1 ml of trypsin (Gibco-BRL) 2 mg/ml solution, pH 7.8, incubated with shaking at 37°C for 45 min, centrifuged and resuspended in PBS. After further centrifugation, pelleted cells were resuspended in bovine serum albumin (BSA; Sigma) 5 mg/ml in PBS.

HT29 cell culture

HT29 cells were grown in culture flasks in Dulbecco's medium with glutamine, supplemented with penicillin 100 IU/ml, streptomycin 100 $\mu\text{g}/\text{ml}$ and fetal bovine serum 10% (hereafter referred to as medium), at 37°C in air with CO_2 5%. Cells were transferred to fresh medium twice a week.

Adherence to HT29 cells

Radiolabelled bacteria were prepared by growth in 5 ml of BHI-PRAS containing S^{35} methionine (100 $\mu\text{Ci}/\text{ml}$). The cultures were incubated anaerobically for 24 h at 37°C . HT29 cell-culture monolayers were prepared in Falcon multiwell trays (2 cm^2 wells). Confluent cell layers were washed twice with PBS immediately before use. Radiolabelled bacteria were washed three times with PBS and resuspended to OD_{550} 0.4

(10^9 bacteria/ml). Bacterial suspension (0.5 ml) was added to each of the monolayers. Trays were shaken on a plate shaker for 150 min at room temperature. The unbound bacteria were removed by washing the cells three times with PBS; finally, PBS (0.5 ml) was added to each well. All cells were lysed with 1.5 ml of sodium dodecyl sulphate (SDS) 5.5% w/v overnight at room temperature. The amount of radioactivity was determined by liquid scintillation counting. The radioactivity bound in the assay was compared with the total count in unwashed cells to give a percentage adherence value [3].

Inhibition of adherence assays

The reference strain ATCC 25285 was radiolabelled and treated with heat, trypsin and EDTA as described above in the haemagglutination inhibition assays. The adherence assay was done with and untreated suspension as control.

Hydrophobicity level

Organisms were grown in BHI-PRAS [11] for 18 h at 37°C and then washed three times with PBS and resuspended to OD_{660} 0.4. Xylene 1:2.5 (v:v) was added to the bacterial suspension, shaken in a 'vortex' mixer for 2 min, and then kept at room temperature for 20 min. The OD_{660} of the lower phase was determined. The hydrophobicity level (HL) was calculated from the formula:

$$\text{HL} = \frac{0.4 - \text{OD}}{0.4} \times 100\% \quad [13].$$

The isolates with percentages $> 50\%$ were considered hydrophobic.

Fragilysin activity

Organisms were grown overnight in BHI-PRAS at 37°C . The cultures were centrifuged at 4000 g for 10 min and the supernates were frozen immediately and kept at -20°C until used. HT29 cells from one flask were resuspended in 20 ml of medium, distributed into a 96-well microtitration plate (200 $\mu\text{l}/\text{well}$) and allowed to grow for 2–3 days. Before the assay the medium was removed and 180 μl of fresh medium without serum were added to each well: 20 μl of bacterial culture supernates were inoculated into the wells in quadruplicate. The plate was incubated at 37°C in air with CO_2 5% and then examined after 2 and 4 h. The culture supernates were considered to contain fragilysin if a cytopathic effect was visible [14].

Enterotoxin activity in the ligated intestinal loop assay

B. fragilis supernates from cultures in log phase (HT29⁺ and ⁻) were injected into loops in the proximal ileum of adult rabbits, which were then killed

after 18 h and examined for the secretory response in the ligated loops. Sterile BHI and PBS were used as negative controls and the *E. coli* strain 40T (LT⁺) as a positive control [15].

Detection of fragilysin gene (*bft*)

Pure genomic DNA from all strains tested was obtained by a standard miniprep procedure [16]. The concentration of DNA in the samples was determined by spectrophotometry with the GenQuant apparatus (Pharmacia). PCR was used to obtain the DNA fragment of the *bft* gene, with the specific primers: 5'-TGTGGAATCA-CATCGTGCATCA-3' and 5'-AGTTCGCCGCAT-CCTGCATCTG-3' (Gibco-BRL). PCR was performed with *Taq* polymerase 1.25 U (Gibco-BRL) in 50 µl containing chromosomal DNA (50 ng), primers (80 pmol), deoxynucleoside triphosphates (200 µM) and 1.5 mM MgCl₂. The amplification cycle was denaturation at 94°C for 1 min, annealing at 66°C for 2 min and extension at 72°C for 5 min [17]. The amplified product was analysed by agarose gel electrophoresis.

Results

Different adhesion patterns were detected in the haemagglutination and auto-agglutination assays with the 58 isolates (Tables 1 and 2): HA⁺ AA⁻ strains (18 isolates), HA⁺ AA⁺ strains (13), HA⁻ AA⁺ strains (1) and HA⁻ AA⁻ strains (26). However, when BSA was used in the inhibition tests, these patterns were altered, as in many cases the inhibition of AA determined the loss of HA (strains 1103-4 and 1118-1 in Table 1 and strains 0783220 and Bd1 in Table 2). Based on these results the groups were re-arranged: HA⁺ AA⁻ strains

(18), HA⁺ AA⁺ strains (9), HA⁻ AA⁺ strains (1) and HA⁻ AA⁻ strains (30). Treatment with trypsin, heat and EDTA inhibited HA and AA of all strains. In the assays for determination of surface hydrophobicity, 15 of 22 isolates from non-intestinal infections were defined as hydrophobic and seven as hydrophilic (Table 1). In the group of 36 isolates from intestinal infections, intestinal microflora and from polluted aquatic environment 22 isolates were defined as hydrophobic and 14 as hydrophilic (Table 2).

When a selected group of nine isolates from different sources, without AA and with different HA titres, was assayed with HT29 cells, the percentage of adherence ranged from 7.7% to 30% (Table 3). The number of adhering bacteria/cell was calculated for each isolate and isolates with <150 bacteria/cell were considered poorly adherent (0), moderately adherent isolates (3) had 150–300 bacteria/cell and highly adherent strains (6) had >300 bacteria/cell.

Treatment with trypsin, heat and EDTA inhibited the cell adhesion capacity of strain ATCC 25285.

In all 58 isolates analysed, it was possible to verify that only two isolates originating from diarrhoea samples (strains 078320 and 079298-3) were able to express fragilysin in assays with HT29 cells, causing rounded cells after incubation for 2–4 h. The other isolates analysed originated from non-intestinal infections, human intestinal microflora, animal intestinal microflora, polluted aquatic environment, animal intestinal infections and also human intestinal infections (strains 283-2-1 and 077225-2) did not show cytotoxicity to HT29 cells. The supernates of strains 078320 and 079298-3 were serially diluted in Dulbecco's medium

Table 1. Determination of haemagglutination (HA), auto-agglutination (AA) and surface hydrophobicity (SH) in *B. fragilis* isolates from non-intestinal infections

Source	Isolate no.	HA titre	HA BSA	AA	AA BSA	SH
ATCC reference	25285	0	NT	0	NT	+
	23745	4	4	0	0	+
Chronic otitis media	036M	0	NT	0	NT	+
	016M	2	2	1+	1+	+
	023A	4	4	0	0	–
	1058-1	0	NT	0	NT	–
Intra-abdominal infections	1031, 1304-3a	2	2	0	0	–
	1304-2	8	8	0	0	+
	1103-4	2	0	4+	0	+
	1048-a	8	8	3+	3+	+
Bacteraemia	1081	2	2	0	0	+
	1032	2	2	0	0	–
	1241	8	8	3+	3+	+
	1097	0	NT	0	NT	+
Skin and soft tissue infections	1108-3b	0	NT	0	NT	–
	MC1	2	2	0	0	–
	1118-1	64	0	4+	0	+
	MC2, 1292	0	NT	0	NT	+
Miscellaneous	1034	2	NT	0	NT	+
	1270-3a1	8	8	3+	3+	+

HA titre, highest bacterial dilution that showed agglutination of human erythrocytes A+; HA BSA, haemagglutination titre in the presence of BSA 5 mg/ml; AA, bacterial suspension only, evaluated on a scale from 1+ to 4+; AA BSA, auto-agglutination evaluated with BSA 5 mg/ml; SH, phase partition in xylene, > 50% hydrophobic (+), < 50% hydrophilic (–); NT, not tested.

Table 2. Determination of haemagglutination (HA), auto-agglutination (AA) and surface hydrophobicity (SH) in *B. fragilis* isolates from human intestinal infections, animal intestinal infections, human intestinal microflora, animal intestinal microflora and polluted aquatic environment

Source	Isolate no.	HA titre ¹	HA BSA	AA	AA BSA	SH
Human intestinal microflora	108311, Re51,	0	NT	0	NT	+
	F4, F26-6	0	NT	0	NT	+
	FF15, F22, FN17-80	0	NT	0	NT	-
	F1, FF10,	2	2	0	0	+
	FF14, F43-6	2	2	0	0	+
	F21	4	4	0	0	+
	Re32	4	4	3+	3+	+
	118310	2	2	0	0	-
	C102b	0	NT	0	NT	+
	P1, P2, P3	0	NT	0	NT	-
Animal intestinal microflora	C102a	4	4	0	0	-
	078320	2	0	1+	0	-
	079298-3, 077225-2	0	NT	0	NT	+
Human intestinal infection	283-2-1	0	NT	0	NT	-
	Bd1	2	0	4+	0	-
	865193-4	16	16	4+	4+	-
Polluted aquatic environment	AA10	2	2	0	0	-
	AA41-1	2	2	1+	1+	-
	AA41-4	0	0	1+	0	+
	AA1, AA41-3,	0	NT	0	NT	+
	AA41-4, AA42-1	0	NT	0	NT	+
	AA42-2	0	NT	0	NT	+
	AA3	8	8	0	0	+
	AAa	8	8	3+	3+	+
	AA7	16	16	4+	4+	+

See footnote to Table 1.

Table 3. Determination of adherence to HT29 cells by *B. fragilis* isolates from non-intestinal infections, human intestinal infections, human intestinal microflora and polluted aquatic environment, with and without HA

Source	Isolate nos.	HA titre	SH	Adherence (%) [*]	Number of bacteria/cell [†]
Extraintestinal infections	25285 [‡]	0	55	21.9	548
	023A [§]	4	45	15.5	388
	1304-2	8	70	15.6	390
Human intestinal infections	0772252 [*]	0	55	14.2	355
	079298-3 ^{**}	0	55	10.6	265
	078320 ^{**}	2	45	7.8	195
Human intestinal microflora	FF15	0	50	20.3	508
Polluted aquatic environment	AA1	0	50	30.0	750
	AA3	8	50	11.0	275

HA, SH, see footnote to Table 1.

^{*}percentage adherence, difference between total radioactivity in the inoculum added and radioactivity bound in the assay.[†]number of bacteria/cell, calculated from estimated number of cells/well (2×10^5 in 2 cm^2) and the bacterial inoculum (1×10^8), related to the percentage adherence.[‡]ATCC reference strain.[§]From chronic otitis media.^{||}From intra-abdominal infection.^{*}Non-enterotoxigenic strain.^{**}Enterotoxigenic strains.

without fetal serum, and the cytotoxic titres were 8 and 128, respectively (Table 4).

The enterotoxic activity of fragilysin was also confirmed in the rabbit intestinal ligated loop assay when the supernates of the cytotoxic strains were assayed.

The presence of the fragilysin gene was confirmed by PCR with specific primers for the *bft* gene; all 58 strains were assayed and 900-bp bands in agarose gel electrophoresis were found only in the cytotoxic strains 078320 and 079298-3 (Fig. 1).

Discussion

In this study, the HA technique was used to characterise the adherence properties of isolates, considered to represent the factor that may contribute to the prevalence of *B. fragilis* in infections. The capacity for HA and AA have been associated with more virulent strains [12]. These mechanisms have not yet been explained in molecular terms, although a proteic nature for the molecules involved has been suggested [18]. A direct relationship between the surface hydrophobicity and both approximation and interaction

Table 4. Determination of cytotoxic activity for HT29 cells and detection of *bft* gene by PCR

Isolate no.	Cytotoxic titre (HT29)	<i>bft</i> gene (PCR)*
25285, 23745 [†]	0	—
078320 [‡]	8	+
079298-3 [‡]	128	+
283-2-1 [‡]	0	—
077225-2 [‡]	0	—
Other isolates	0	—

*PCR product with specific primers.

[†]ATCC reference strains.[‡]Strains given by Dr Lyle Myers, Montana University, EUA, from human intestinal infection, as positive (078320, 079298-3) and negative (283-2-1, 077225-2) controls of enterotoxin production in lamb ligated intestinal loop assay. All other 52 isolates were tested by both assays and did not have either cytotoxic activity or *bft* gene presence. **Fig. 1.** Detection of *bft* gene by PCR analysis and agarose (1.0%) gel electrophoresis. Bands of c. 900 bp were detected only in the two isolates from diarrhoeic sources (arrow). Line a, mol. wt standard (Gibco, 1 kb); b, DNA amplified from strain 079298-3; c, 283-2-1; d, 077225-2; e, ATCC 25285; f, ATCC 23745; g, AA1; h, Bd1; i, FF15; j, 078320.

between bacteria and other surfaces has been proposed [19]. In the present study, HA was totally inhibited by physicochemical treatments, suggesting that the haem-agglutinins present have a proteic nature and an activity dependent on metallic ions. Bovine serum albumin was capable of inhibiting AA and HA in the isolates studied; however, hydrophobicity and the origins of isolates do not seem to be related to AA and HA.

Adherence by *B. fragilis* has also been analysed in other cell culture systems. Oyston and Handley [3] verified lower levels of adhesion of buccal epithelial cells than to intestinal epithelial HT29 cells. In testing

the adherence of *B. fragilis* to HT29 cells, it was verified that isolates with higher titres of HA were less adherent to the cells, which could suggest the existence of different molecules responsible for the two features. Thus, HA determination was not satisfactory as a selection criterion for isolates adherent to intestinal epithelial cells (HT29). Treatment with heat, trypsin and EDTA produced significant reduction in the adhesion of isolates to HT29 cells, suggesting a proteic nature for both molecules involved. The surface hydrophobicity levels did not correspond with the adherence properties demonstrated, suggesting selectivity in the adhesion. There was no correlation between adherence properties and the sources of isolates; consequently, it was not possible to propose them as virulence markers.

Only two *B. fragilis* isolates from intestinal infections expressed fragilysin, and there was complete correlation between phenotypic (HT29) and genotypic (PCR) methods of detection. The PCR assay proved to be a good methodological choice for fragilysin detection, due to its speed and ease of reading. The evaluation of enterotoxin activity by rabbit ligated intestinal loop assay was successful; however, some authors do not recommend this experimental animal model, because it is less sensitive than the lamb ligated intestinal loop [20].

The fragilysin molecule was characterised as a zinc-metalloprotease [8] that induces alterations in the intestinal epithelial cells, mainly in the tight junction proteins with disruption of the epithelial barrier [21]. The histological responses suggest a role for fragilysin as a factor that could act in the initial stages of non-intestinal infectious processes caused by *B. fragilis*. Kato *et al.* [10] proposed that enterotoxigenic *B. fragilis* (ETBF) was preferentially associated with bacteraemia. It has also been shown that the presence of the toxin increased the internalisation of enteric bacteria, such as *Salmonella typhimurium* and *E. coli* by HT29 cells [22], leading to the belief that fragilysin may contribute to the disruption of the barrier against non-intestinal infections. In the present study, the isolates from invasive infections did not produce fragilysin, as would be expected. There was a correlation between isolates from intestinal infections and the presence of fragilysin. Mundy and Sears [23] also found a small number of ETBF in non-intestinal infections. Thus, it seems too early to suggest this protease as a determinant factor of *B. fragilis* invasiveness, and more studies are needed.

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